Note

Sesquiterpenes from the Fruiting Bodies of *Ramaria formosa* and Their Human Neutrophil Elastase Inhibitory Activity

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Two new sesquiterpene derivatives (**1** and **2**), ramarin A (**1**) and ramarin B (**2**), together with three known compounds (**3**–**5**) were isolated from the fruiting bodies of *Ramaria formosa*. The structures of the two sesquiterpenes were established by extensive spectroscopic studies and chemical evidence. The inhibitory activity of the isolated compounds (**1**–**5**) against human neutrophil elastase (HNE) was evaluated *in vitro*. All compounds tested inhibited HNE by 35–30% at the highest concentration used (100 μ M), whereas the positive control, epigallocatechin gallate (EGCG), exhibited 60% inhibition at 100 μ M.

Key words *Ramaria formosa*; Gomphaceae; sesquiterpene; human neutrophil elastase

Human neutrophil elastase (HNE), a serine protease found primarily in the azurophilic granules of neutrophils, has broad substrate specificity and can cleave not only elastin but also other extracellular matrix (ECM) proteins, such as collagen, fibronectin, laminin, and proteoglycans.¹⁾ With aging, skin elasticity is decreased significantly by elastase activity, resulting in skin sagging and wrinkling.²⁾ Thus, developing pharmacological inhibitors of HNE might provide a therapeutic approach for preventing skin sagging and wrinkling in aging.

Many species of mushrooms have been used in folk medicine around the world for thousands of years. They produce various classes of secondary metabolites, many of which possess interesting biological activities and have the potential to be developed as therapeutic agents.^{3–5)} Thus, mushrooms have been the focus of much attention in the search for specific pharmacologically significant compounds.

Ramaria formosa (PERS.) QUÉL. is a coral fungus of the genus *Ramaria* in the family Gomphaceae, found primarily in Europe and North America. Previous studies revealed that the crude extract of *R. formosa* exhibited antimicrobial, anti-oxidant, and antitumor activities.^{6,7)} Cellulase activity was also detected in a culture of *R. formosa*.⁸⁾ Recently, a novel ribonuclease purified from *R. formosa* was reported to exhibit human immunodeficiency virus (HIV)-1 reverse transcriptase inhibitory activity.⁹⁾ However, there is no reported study of the secondary metabolites of this mushroom.

In searching for novel HNE inhibitors from higher fungi, we found that an ethyl acetate (EtOAc)-soluble fraction of the methanol (MeOH) extract of the fruiting bodies of *R. formosa* had considerable HNE-inhibitory activity (IC₅₀ = 40.6 μ g/mL). Further studies on this fraction resulted in the isolation of two new sesquiterpene derivatives (**1** and **2**), together with three known compounds (**3**–**5**). This report describes the isolation and structural determination of these sesquiterpene derivatives, and the characterization of their inhibitory effects against HNE.

Results and Discussion

MeOH extract of the fruiting bodies of *R. formosa* was suspended in H₂O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc-soluble fraction, which inhibited HNE activity significantly, was subjected to a series of chromatographic procedures, leading to the isolation of two new sesquiterpene derivatives (**1** and **2**), together with three known compounds (**3**–**5**) (Fig. 1). The three known compounds were identified as nambinone A (**3**),¹⁰⁾ axinysonone A (**4**),¹¹⁾ and *ent*-aristolone (**5**)¹²⁾ and by comparing their physicochemical and spectral data with those in the literature.

Compound **1** was obtained as a colorless oil with the molecular formula C₁₅H₂₂O₃, as established by high resolution-electron ionization-mass spectrum (HR-EI-MS), based on a molecular ion peak at *m/z* 250.1567 [M]⁺. The EI-MS of **1** showed a parent peak at *m/z* 250 and characteristic fragment

Fig. 1. Chemical Structures of **1**–**5** Isolated from the Fruiting Bodies of *R. formosa*

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Table 1. ^1H - (300 MHz) and ^{13}C - (75 MHz) NMR Data of Compounds **1** and **2** in MeOD

C	1		2	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	69.7 d	4.38 dd (12.0, 5.2)	31.8 t	ax: 2.56 ddd (13.4, 12.2, 3.4), eq: 2.32 ddd (13.4, 3.6, 1.8)
2	36.3 t	ax ^a : 1.40 dddd (13.5, 12.0, 12.0, 5.2), eq ^b : 2.10 m	34.5 t	ax: 1.86 dddd (13.4, 12.2, 12.2, 3.4), eq: 1.42 m
3	29.5 t	ax: 1.48 dddd (13.4, 13.0, 12.2, 3.2), eq: 1.62 m	27.4 t	ax: 1.58 dddd (13.4, 12.2, 12.2, 3.4), eq: 1.50 m
4	39.8 d	1.85 dqd (12.2, 6.8, 3.6)	40.2 d	1.88 dqd (12.2, 6.5, 3.4)
5	40.6 s		40.7 s	
6	37.0 d	1.65 d (8.0)	36.5 d	1.66 d (8.0)
7	32.3 d	1.83 d (8.0)	32.7 d	1.83 d (8.0)
8	199.2 s		199.1 s	
9	121.3 d	6.19 brs	125.0 d	5.74 brs
10	173.3 s		172.4 s	
11	31.0 s		31.8 s	
12	71.2 t	3.42 d (11.6), 3.32 d (11.6)	71.2 t	3.43 d (11.6), 3.36 d (11.6)
13	12.5 q	1.23 s	12.7 q	1.23 s
14	24.0 q	1.24 s	23.3 q	1.27 s
15	16.2 q	1.10 d (6.8)	16.6 q	1.10 d (6.8)

a) Axial proton. b) Equatorial proton.

ions at m/z 232 ($[\text{M}-\text{H}_2\text{O}]^+$) and 217 ($[\text{M}-\text{H}_2\text{O}-\text{CH}_3]^+$). The UV absorption maxima at 239 nm and the IR absorption band at 1665 cm^{-1} suggested the presence of a conjugated keto group, which was supported by the presence of a carbon resonance at δ_{C} 199.2 in the ^{13}C -NMR spectrum. The ^1H -NMR spectrum of **1** displayed the characteristic signals of an olefinic proton at δ_{H} 6.19 (1H, brs), a hydroxymethine proton at δ_{H} 4.38 (1H, dd, $J=12.0, 5.2\text{ Hz}$), a pair of hydroxymethylene protons at δ_{H} 3.42 and 3.32 (2H, each d, $J=11.6\text{ Hz}$), two tertiary methyls at δ_{H} 1.23 and 1.24 (each 3H, s), and a secondary methyl at δ_{H} 1.10 (3H, d, $J=6.8\text{ Hz}$) (Table 1). The ^{13}C -NMR spectrum, combined with the distortionless enhancement by polarization transfer (DEPT) data, revealed 15 carbon signals including a carbonyl carbon at δ_{C} 199.2, olefinic carbons at δ_{C} 173.3 and 121.3, a hydroxymethine at δ_{C} 69.7, a hydroxymethylene at δ_{C} 71.2, and three methyls at δ_{C} 24.0, 16.2, and 12.5 (Table 1). These spectroscopic data suggested **1** to be an aristolane-type sesquiterpene, in comparison with reported analogs.^{10–12} Additionally, the ^1H - and ^{13}C -NMR data of **1** were very similar to those of axinyson A (**4**), except for the resonances of a hydroxymethylene unit (δ_{H} 3.42 and 3.32; δ_{C} 71.2) instead of a methyl group at C-12 (δ_{H} 1.21; δ_{C} 29.7) in **4**, suggesting that the C-12 methyl group of **4** was replaced by a hydroxymethylene group in **1**. This was supported by the ^{13}C -NMR chemical shift of C-11 (δ_{C} 31.0), as well as analysis of the heteronuclear multiple bond connectivity (HMBC) spectrum (Fig. 2A). The HMBC correlations H-6/C-7, C-8, C-9 and H-7/C-6, C-8, C-9, C-10 confirmed that a cyclopropane moiety was connected to C-6 and C-7. The attachment of a hydroxymethylene group at C-11 was deduced from the HMBC cross-peaks of H-12 with C-6, C-7, C-11, and C-13. The relative configuration of **1** was determined on the basis of the nuclear Overhauser effect spectroscopy (NOESY) experiment (Fig. 2B) and a comparison with that of axinyson A (**4**). The β -configurations of the methyl groups at C-14 and C-15 were deduced from the nuclear Overhauser effect (NOE) correlations Me-14/H-3ax and H-4ax/H-2ax, respectively.

The NOE correlations H-6/Me-14 and H-4/Me-13 indicated an α -configuration of the cyclopropane. Finally, the NOE correlations H-1/H-3ax, Me-14 lead to the assignment of an α -configuration of the hydroxy group at C-1, as found in **4**. The relative configuration of **1** was assigned as being identical to that of **4** on the basis of the NOE correlations. The absolute configuration of **1** was assumed to be the same as that of *ent*-aristolone series, possessing the $4S,5S,6R,7S$ configuration, from the positive sign of its specific rotation, $[\alpha]_{\text{D}}^{25}+180^\circ$ (c 0.1, MeOH), and its co-occurrence with **3–5** that possess an *ent*-aristolone structure.^{10–12} Thus, the structure of **1** was established as *ent*-1 α ,12-dihydroxy-9-aristolen-8-one, and named ramarin A.

Compound **2** was obtained as a colorless oil, and showed a molecular ion peak at m/z 234.1622 $[\text{M}]^+$ in HR-EI-MS, corresponding to the molecular formula $\text{C}_{15}\text{H}_{22}\text{O}_2$. The UV ($\lambda_{\text{max}}=237\text{ nm}$) and IR ($\nu_{\text{max}}=1662\text{ cm}^{-1}$) spectra of **2** were characteristic of a conjugated keto group. The ^1H -NMR spectrum of **2** was very similar to that of *ent*-aristolone (**5**), with the appearance of two typical high-field doublets at δ_{H} 1.66 and 1.83 (each 1H, d, $J=8.0\text{ Hz}$) as well as a singlet olefinic proton at δ_{H} 5.74 (Table 1), suggesting that **2** had the same aristolane skeleton with a cyclopropane group. The difference in the ^1H -NMR spectrum of **2** versus that of **5** was the appearance of signals at δ_{H} 3.43 and 3.36 (each 1H, d, $J=11.6\text{ Hz}$), assignable to hydroxymethylene protons instead of the 12-methyl protons in **5**. Inspection of the ^{13}C -NMR spectrum in comparison with **5** revealed a marked downfield shift of the C-11 signal (δ_{C} 31.8) (Table 1), suggesting the presence of a hydroxymethylene group at C-11 in **2**, further supported by the HMBC correlations of H-12 with C-6, C-7, C-11, and C-13 (Fig. 2A). The relative configuration of **2** was deduced as being identical to that of **5** from the NOESY spectrum (Fig. 2B). Furthermore, the positive sign of its specific rotation, $[\alpha]_{\text{D}}^{25}+93^\circ$ (c 0.1, MeOH), indicated that **2** possess an *ent*-aristolone structure, as found in **3–5**. Thus, the structure of **2** was assigned as *ent*-12-hydroxy-9-aristolen-8-one, and named

Fig. 2. Key ^1H - ^1H COSY and HMBC (A) and Key NOE (B) Correlations of Compounds **1** and **2**

Fig. 3. HNE-Inhibitory Activity of **1**–**5** Isolated from the Fruiting Bodies of *R. formosa*

Results are expressed as the means $\pm$ S.D. of three individual experiments. * p <0.05, ** p <0.01 vs. Control.

ramarin B.

The inhibitory activity of the isolated compounds (**1**–**5**) on HNE was evaluated *in vitro* according to a procedure described previously.¹³⁾ As shown in Fig. 3, HNE activity was reduced by treatment with **1**–**5** in a dose-dependent manner. However, all tested compounds inhibited HNE by 35–30% at the highest concentration used (100 μM), whereas the positive control, epigallocatechin gallate (EGCG), exhibited 60% inhibition at 100 μM .

In the present study, we sought to identify effective HNE-inhibiting agents from the fruiting bodies of *R. formosa*. We isolated two new (**1** and **2**) and three known aristolane

sesquiterpenes (**3**–**5**). This is the first report of secondary metabolites from *R. formosa*. Aristolane-type sesquiterpenes have been reported to exhibit a number of biological activities, including cytotoxic,^{10,14)} antimicrobial,¹⁵⁾ and melanin synthesis inhibition^{16,17)} properties. Although the aristolane sesquiterpenes (**1**–**5**) isolated in this study did not show marked inhibitory effects on HNE, their HNE-inhibitory properties are reported here for the first time.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO P-2000 digital polarimeter. UV

and IR spectra were recorded on JASCO V-550 UV/Vis and JASCO 100 IR spectrometers, respectively. ^1H - (300 MHz) and ^{13}C -NMR (75 MHz) spectra were obtained using a Bruker DRX-300 spectrometer with TMS as an internal standard. Two dimensional (2D)-NMR experiments (correlation spectroscopy (COSY), HMBC, and NOESY) were run on a Bruker Avance 500 NMR spectrometer. HR-EI-MS and EI-MS were recorded on a Hewlett-Packard 5989B mass spectrometer. Column chromatography was performed using silica gel (70–230 mesh and 230–400 mesh, Merck) and YMC-gel ODS-A (12 nm, S-75 μm , YMC). Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F₂₅₄ (0.25 mm, Merck) and RP-18 F_{254s} plates (0.25 mm, Merck). Spots were detected by UV light (254 nm) and spraying with 10% H_2SO_4 followed by heating. Preparative HPLC was performed on a Gilson TRILUTION system with a 321 pump and a UV/Vis 151 detector, and a GX-271 liquid handler (Gilson, Inc.), using a YMC-pack Pro C₁₈ (250 $\times$ 10 mm, i.d.) column.

Mushroom Material Fruiting bodies of *R. formosa* were collected at Mt. Jeoksang, Jeollabukdo, Korea in August 2013 and identified by Prof. KiHwan Bae, Chungnam National University, Republic of Korea. A voucher specimen (CNU-982) was deposited in the herbarium at the College of Pharmacy, Chungnam National University, Republic of Korea.

Extraction and Isolation Dried fruiting bodies of *R. formosa* (350 g) were extracted with MeOH (3 $\times$ 8 L) at room temperature for seven days, filtered, and concentrated to yield a MeOH extract (22 g). This extract (20 g) was suspended in H_2O (1 L) and partitioned successively with *n*-hexane (3 $\times$ 1 L), EtOAc (3 $\times$ 1 L), and *n*-BuOH (3 $\times$ 1 L) to yield *n*-hexane-, EtOAc-, and *n*-BuOH-soluble fractions of 8.0, 1.1, and 5.2 g, respectively. The EtOAc-soluble fraction, which inhibited HNE significantly (IC_{50} = 40.6 $\mu\text{g/mL}$), was subjected to silica gel column chromatography (70–230 mesh, 50 $\times$ 5.0 cm), and eluted with a gradient solvent system consisting of CH_2Cl_2 –MeOH (50:1 $\rightarrow$ 4:1) to generate five fractions (E1–E5). These five fractions inhibited HNE by 72, 68, 52, 43, and 22%, respectively, at 100 $\mu\text{g/mL}$. Thus, fractions E1 and E2 that significantly inhibited HNE were investigated extensively.

Fraction E1 (150 mg) was applied to a YMC RP-18 column (50 $\times$ 3.5 cm), and eluted with a MeOH– H_2O gradient solvent system (3:7 $\rightarrow$ 5:5), yielding two subfractions (E1.1–E1.2). These fractions were further purified by preparative reverse phase (RP)-HPLC [Gilson TRILUTION system; YMC-pack Pro C₁₈ (250 $\times$ 10 mm, i.d.) column; MeOH– H_2O (60:40, v/v); UV detection, 254 nm; flow rate 2.5 mL/min] to afford **4** (0.8 mg, t_{R} = 30.2 min) and **5** (1.8 mg, t_{R} = 32.0 min) from E1.1 and **1** (1.0 mg, t_{R} = 34.0 min) from E1.2. Compounds **2** (5 mg, t_{R} = 31.0 min) and **3** (7 mg, t_{R} = 37.4 min) were isolated from E2 (210 mg) using preparative RP-HPLC [Gilson TRILUTION system; YMC-pack Pro C₁₈ (250 $\times$ 10 mm, i.d.) column; MeOH– H_2O (40:60, v/v); UV detection, 254 nm; flow rate 2.5 mL/min].

Ramarin A (1): Colorless oil. $[\alpha]_{\text{D}}^{25}$ +180° (c = 0.1, MeOH). UV (MeOH) λ_{max} nm (log ϵ): 239 (3.95). IR (KBr) ν_{max} cm^{-1} : 3420, 1665, 1650. EI-MS m/z (rel. int.): 250 [M]⁺ (10), 232 (66), 217 (100), 201 (26), 175 (49). HR-EI-MS m/z : 250.1567 [M]⁺ (Calcd for $\text{C}_{15}\text{H}_{22}\text{O}_3$: 250.1569). ^1H - and ^{13}C -NMR data,

see Table 1.

Ramarin B (2): Colorless oil. $[\alpha]_{\text{D}}^{25}$ +93° (c = 0.1, MeOH). UV (MeOH) λ_{max} nm (log ϵ): 237 (3.92). IR (KBr) ν_{max} cm^{-1} : 3410, 1662, 1654. EI-MS m/z (rel. int.): 234 [M]⁺ (30), 219 (35), 203 (55), 164 (100), 149 (60), 121 (70), 91 (65). HR-EI-MS m/z : 234.1622 [M]⁺ (Calcd for $\text{C}_{15}\text{H}_{22}\text{O}_2$: 234.1620). ^1H - and ^{13}C -NMR data, see Table 1.

HNE Inhibitory Assay HNE inhibitory activity was evaluated according to a previously described procedure.¹³ Briefly, 100 μL reactions containing 10 mM Tris–HCl buffer (pH 7.5), 1.4 mM MeO–Suc–Ala–Ala–Pro–Val–*p*-nitroanilide, 0.18 U HNE (EC 3.4.21.37, from Serva, Heidelberg, Germany), and various concentrations of sample were incubated in the wells of a 96-well plate for 2 h at 37°C in the dark. Each reaction was stopped by the addition of 100 μL soybean trypsin inhibitor (0.2 mg/mL), and the absorbance at 405 nm was immediately measured using a microplate reader. EGCG was used as a positive control.

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Conflict of Interest The authors declare no conflict of interest.

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